NUCLEAR MAGNETIC RESONANCE STUDIES OF REDOX-INDUCED CONFORMATIONAL CHANGES IN THIOREDOXIN FROM *ESCHERICHIA COLI*

Arne HOLMGREN

Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

and

Gordon ROBERTS

National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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1. Introduction

Thioredoxin is a widely distributed hydrogen transport protein which has been purified to homogeneity from bacteria, yeast and bovine liver [1–3]. This small protein operates via a reversible dithiol-disulfide mechanism and carries hydrogen from NADPH to enzymatic reduction processes like the formation of deoxyribonucleotides [4]. Thioredoxin from *Escherichia coli* is composed of a single polypeptide chain having 108 amino acid residues of known amino acid sequence [5]. The three-dimensional structure of oxidized thioredoxin to 2.8 Å resolution by X-ray crystallography [6] has been determined. The functional part of the molecule is formed from two vicinal half-cystine residues (Cys-32 and Cys-35) which in the oxidized protein forms a disulfide, located in a protrusion of the molecule [6].

A localized conformational change in *E. coli* thioredoxin takes place upon chemical or enzymatical reduction of the oxidized form (thioredoxin-S_2) to give the reduced form (thioredoxin-$(SH)_2$ [7]. This conformational change was first demonstrated by the more than 3-fold increase in fluorescence quantum yield of tryptophan emission of the protein upon reduction [7]. It was subsequently shown that the two tryptophan residues (Trp-28 and Trp-31) both are close to the disulfide bridge in the primary structure of the protein [5]. Further studies of the tryptophan fluorescence of *E. coli* and the homologous yeast thioredoxin with a single tryptophan residue [8] indicated that the large increase upon reduction of the *E. coli* protein was a major change in the fluorescence quantum yield of only Trp-28 due to different microenvironment in oxidized and reduced *E. coli* thioredoxin [9,10].

The present paper describes PMR spectra of oxidized and reduced thioredoxin in H_2O and D_2O. The conformational differences of oxidized and reduced thioredoxin appear small at pH above 7.0 but become appreciable at lower pH. The reduced form of thioredoxin was less stable at pH values below 7.0 and aggregated reversibly close to the isoelectric point. Proton nuclear magnetic resonance spectra of thioredoxin in H_2O led to the identification of two resonance peaks from the indole NH proton of the two tryptophan residues of the molecule. Upon reduction of oxidized thioredoxin only one of these two peaks showed a major change in chemical shift while both peaks shifted as a function of pH.

2. Materials and methods

Thioredoxin from *E. coli* B 3 was prepared as described previously (Method 2) [11] and was of better than 98% purity. The sample of thioredoxin (5.7 μmol, 67 mg) to be used in the NMR experi-

ments was lyophilized from 0.2% NH_4HCO_3, pH 8.0 and dissolved in 1.5 ml of 0.1 M sodium phosphate buffer in D_2O, pH* 7.0 and lyophilized again. The dry residue was dissolved in 1.5 ml of D_2O, giving a final concentration of 3.7 mM and transferred to the NMR probe tube. Dioxane was added as an internal reference to give a final concentration of 2 mM.

Determinations of pH were performed with a Radiometer pHM 26 pH-meter and a Beckman combination electrode type 39030. The pH-determination in D_2O solutions is given as pH*, which is the direct pH meter reading, uncorrected for the isotope effect on the glass electrode. pH* was lowered using small aliquots of 2 M DCl and raised by addition of 1.0 M NaOD. pH*-measurements during PMR titration curves of thioredoxin were made before and after the spectrum was obtained and refer to the temperature of the probe, 27.5 ± 0.5°C.

The PMR spectra of thioredoxin were recorded at 100 MHz. Those in D_2O were recorded on a Varian XL-100-15 spectrometer in the Fourier transform mode, those in H_2O on a Varian HA-100 spectrometer coupled to a Biomac computer of average transitions. Field-frequency lock was obtained from the proton resonance of a hexamethyldisiloxane capillary.

Reduction of thioredoxin-S_2 was achieved by the addition of approximately 12-fold molar excess (10 mg) of recrystallized dithiothreitol (Calbiochem. A.G.).

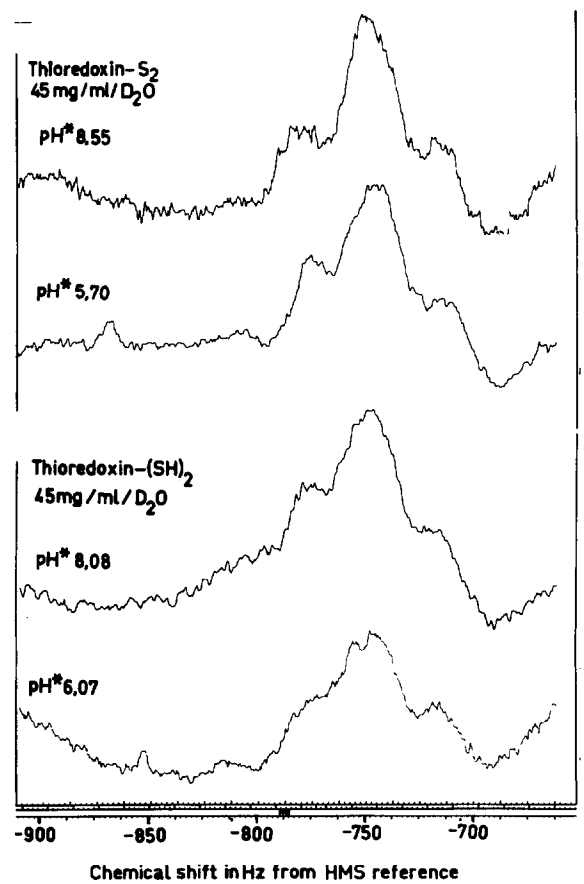

Fig.1. 100 MHz proton NMR spectrum of oxidized and reduced *E. coli*, thioredoxin in 0.1 M sodium phosphate buffer in D_2O, at the indicated pH* values and at 27.5°C. Protein concentration 3.7 mM. Only the aromatic region of the spectra between −6.5 and −9.0 ppm is shown. Each spectrum represents 6000 transients.

3. Results and discussion

3.1. *Proton magnetic resonance spectra in D_2O*

We have here concentrated on the region between −6.5 and −9.0 ppm which contains the protons of the aromatic amino acid residues and histidine-C2 protons [12]. Thioredoxin contains two tryptophan residues (Trp-28 and Trp-31), four phenylalanine residues (Phe-12, Phe-27, Phe-81, and Phe-102), two tyrosine residues (Tyr-49 and Tyr-70) and one histidine residue (His-6). These amino acid residues cover the entire primary structure of the protein, and can thus be regarded as convenient reporter groups of the conformation of various parts of the molecule.

PMR spectra of the aromatic region of thioredoxin-S_2 recorded at different pH*-values are shown in fig.1. The spectrum showed the same overall appearance at pH* 8.55 and pH* 5.70 with the exception of a peak observable at about −8.60 ppm from external HMS. This peak, which changed its chemical shift with pH, was assigned to the C2-proton of His-6. At pH 8.55 the peak moved in under the main aromatic envelope and at pH 4.70 it was found at the extreme low field of the spectrum. From a plot of chemical shift as a function of pH* 8.55 and pH* 5.0, using computer fitting [13] a pK of 5.98 was calculated for this histidine residue. This value is somewhat lower than for an imidazole in a wholly solvent-like environment (see for example King and Roberts, 1971 [13]). From the three-dimensional structure of thioredoxin-S_2 [6],

His-6 is part of a β-pleated sheet strand at the top of the molecule with its side chain exposed, which thus agree with the measured p*K* value.

The spectrum at pH* 4.75 was significantly different from those at higher pH*, and indicated the beginning of a reversible acid unfolding of thioredoxin at this pH*. This acid unfolding of thioredoxin has not been previously observed since tryptophan fluorescence spectra of thioredoxin-S_2 showed the same strongly quenched emission from pH 2 to pH 10 [9]. The isoelectric point of thioredoxin has been determined to be pH 4.5; (1) pH* 4.70 is equivalent to p*D* 5.1. At room temperature, therefore, the acid unfolding of thioredoxin was observed somewhat above the isoelectric point.

A comparison of the proton NMR spectra of thioredoxin-S_2 and chemically reduced thioredoxin-$(SH)_2$ at pH* $\sim$ 8 is shown in fig.1. Only minor differences were noted indicating very similar overall conformations of oxidized and reduced thioredoxin at this pH*, strongly supporting a minor local conformational change upon oxidoreduction. In contrast to thioredoxin-S_2, however, the reduced protein showed spectral shifts of the aromatic region between pH* 8.0 and 5.5 indicating considerably less stability to changes in pH*. At pH* 5.7–6.0 there were clearly appreciable differences in the spectra of the two forms. This is in agreement with the marked changes in tryptophan fluorescence observed for reduced thioredoxin at pH 2.0–8.0 [9].

At pH* 4.60 the spectrum of thioredoxin-$(SH)_2$ was seriously broadened and uncharacteristic indicating considerable aggregation. At the same time the solution of the protein turned cloudy. By titrating the solution back to pH 9.0 the cloudiness disappeared and the spectrum of the aromatic region was again identical with that previously observed at pH* 8.08. These results which demonstrate a reversible aggregation of thioredoxin-$(SH)_2$ at acidic pH are in agreement with previous results from ultracentrifugation studies [1] of *p*-mercuribenzoate-treated thioredoxin-$(SH)_2$. Earlier attempts to reverse the aggregation at pH 7.0 resulted in partial deaggregation [1]; obviously pH 9.0 is necessary to complete this process. The unfolding and aggregation at low pH made it impossible to determine the p*K* of His-6 in the reduced protein. Comparison of its position in the spectra of the two forms at pH* $\sim$ 6, however, suggested little change in p*K*.

The results of NMR-studies here indicate considerable differences in the conformation of oxidized and reduced thioredoxin at pH-values close to the isoelectric point. This result is important, since attempts to obtain crystals of the reduced form of thioredoxin by diffusing thiols into preformed crystals at 4°C close to pH 4.7 ([14] Holmgren, A., unpublished results) have led to destruction of the crystals. If the conformational differences between oxidized and reduced thioredoxin are large at low pH, they might not be positioned in the same crystal lattice.

3.2. *Proton magnetic resonance spectra in H_2O*

In the proton magnetic resonance spectra of thioredoxin-S_2 and thioredoxin-$(SH)_2$ in H_2O, two separate peaks corresponding to indole NH resonances of tryptophan could be identified between −11.0 and −12.0 ppm from external HMS (fig.2). The chemical

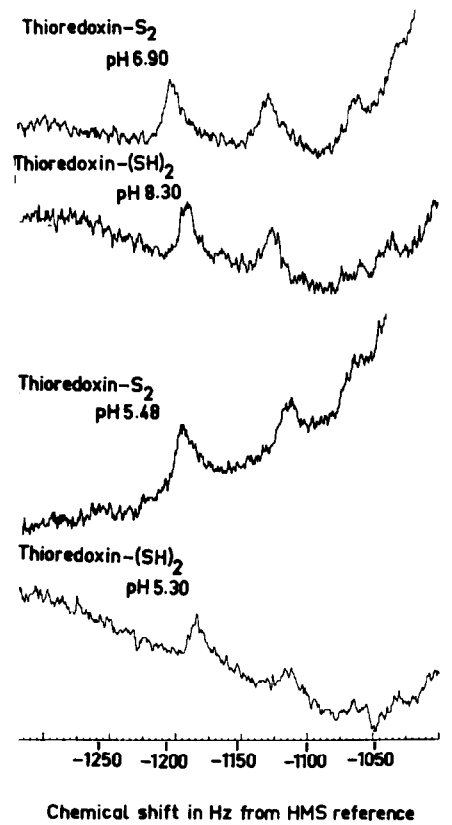

Fig.2. 100 mHz proton NMR spectra of thioredoxin-S_2 and thioredoxin-$(SH)_2$ in H_2O at different pH values. Chemical shifts refer to Hz downfield of external HMS.

Table 1
Chemical shifts of NH-indole peaks of thioredoxin in H_2O[a]

	Low field peak	High field peak
Thioredoxin-S_2, pH 6.90	- 1194 Hz	- 1121 Hz
Thioredoxin-$(SH)_2$, pH 8.30	- 1182 Hz	- 1117 Hz
Thioredoxin-S_2, pH 5.48	- 1186 Hz	- 1107 Hz
Thioredoxin-$(SH)_2$, pH 5.30	- 1176 Hz	- 1106 Hz

[a]Chemical shifts are expressed as Hz downfield from external HMS.

shifts of the resonances are summarized in table 1. The low field peak at −11.94 ppm from HMS in thioredoxin-S_2 moves approximately 0.12 ppm upfield upon reduction to thioredoxin-$(SH)_2$. The high field peak at −11.21 ppm in thioredoxin-S_2 on the other hand moves only 0.04 ppm upon reduction.

When spectra of thioredoxin-S_2 and thioredoxin-$(SH)_2$ were recorded at lower pH* both the high field and low field peaks had moved approximately 0.1 ppm downfield. Again at the lower pH*, reduction of thioredoxin-S_2 leads to an upfield shift of 0.10 ppm of the resonance at lower field, while that at −11.06 ppm is unaffected.

These observations indicate that the two tryptophan residues of thioredoxin, though very close in the sequence, have quite distinct environments in both the oxidized and reduced protein. Reduction of thioredoxin-S_2 affects one of the peaks appreciably more than the other; it is tempting to suggest that the local conformational change in thioredoxin on reduction changes the environment around one of the tryptophan residues. It is not known if this residue is the same as that observed by the fluorescence spectroscopy [9,10]. It is notable that the chemical shifts of both these two indole NH resonances are equally pH-dependent, and that both oxidized and reduced forms of the protein show essentially the same pH dependence. This is in contrast to the finding, discussed above, that the pH-dependence of the overall conformation is quite different in the two forms. From the three-dimensional structure of thioredoxin-S_2 [6], Trp-31 is part of a protrusion formed by residues 29–37 containing the oxidation reduction active disulfide bond. Trp-31 is completely exposed to solvent whereas the other tryptophan residue, Trp-28 is partly shielded by a pleated sheet. The fluorescence of both tryptophan residues have been proposed to be quenched by the disulfide bond in thioredoxin-S_2 [9]. The exact nature of the conformational change occurring in the active site protrusion upon reduction of thioredoxin-S_2 is not known. Further NMR studies of chemically modified thioredoxin [10] may be valuable to define more closely the nature of the conformational change which is part of the oxidation–reduction function in the thioredoxin-molecule.

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